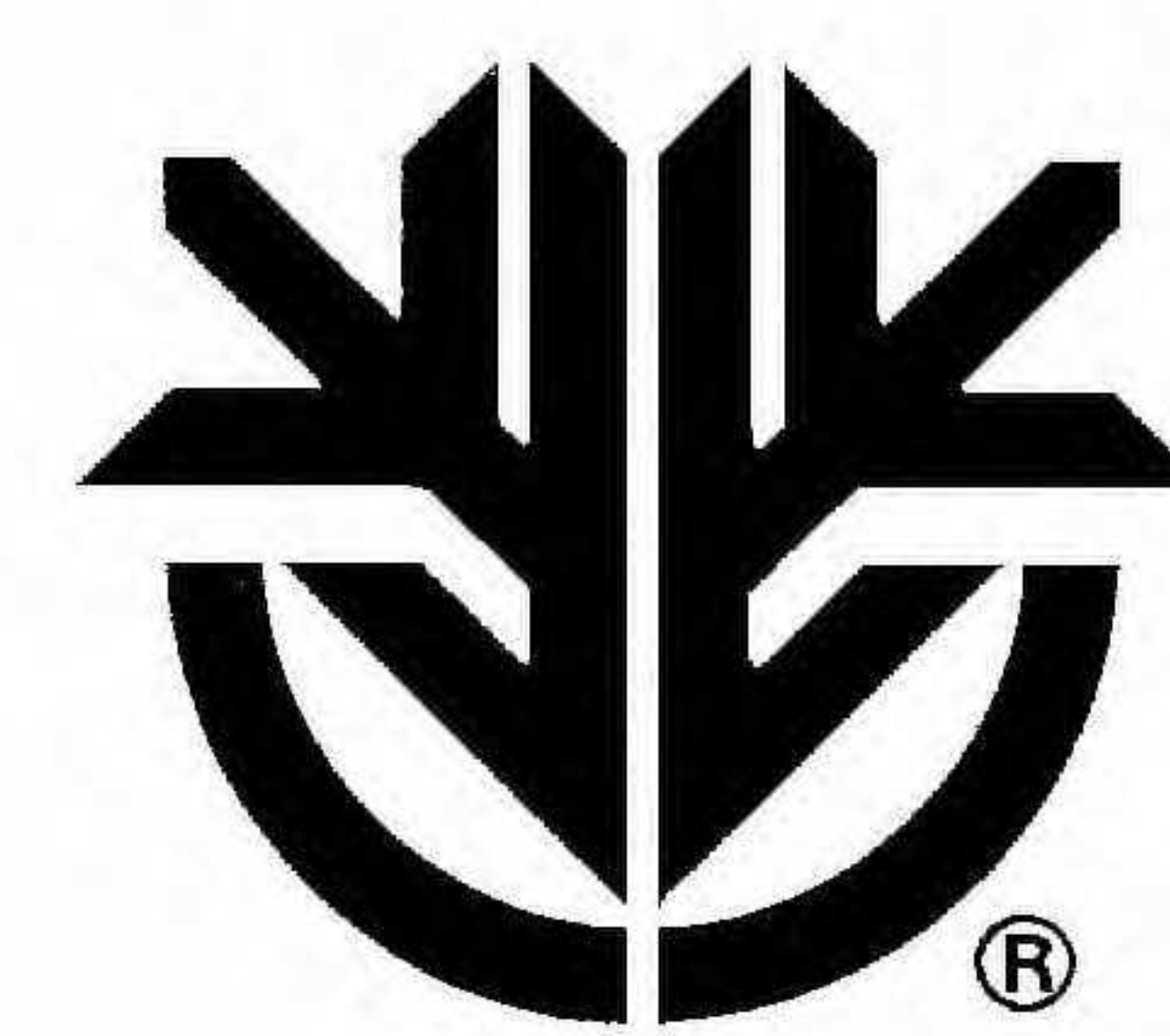

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EVODEVO AND THE PROMISE OF
UNDERSTANDING
MORPHOLOGICAL TRANSITIONS
IN EVOLUTION¹

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ABSTRACT

The field of Evolutionary Developmental biology arose with the promise of new approaches to answering longstanding questions of comparative biology. Here we review the fruits of that promise some decades later. We chose three areas of arthropod EvoDevo—evolution of body plans, segment number, and appendage morphology—to provide an overview for the nonspecialist of how these issues have been clarified by the comparative analysis of regulatory gene networks. In all cases, we identify substantial progress and novel insights provided by the tools and perspective of EvoDevo. We also recognize that some core questions remain unanswered, and we reflect on how discoveries in EvoDevo fit in the landscape of other progress in phylogenetics, population biology, and genomics, facilitated by a new and ever-expanding set of molecular tools for comparative studies in evolution.

Key words: Appendage development, arthropods, development, evolution, segmentation.

What meets the eye when we cursorily inspect nature is an overwhelming variety of morphological forms. One current strategy in biology to explain how diverse forms might have evolved is to compare the regulation of body patterning during development. If we can grasp how form develops among a number of related species, we can hypothesize how modifications in development create distinct morphological forms over evolutionary time. The contemporary study of how developmental patterning evolves—EvoDevo—relies primarily on understanding the gene regulatory pathways that modulate development. At the same time, EvoDevo draws on longstanding intellec-

tual enterprises in science. Here, we briefly describe the development of EvoDevo as a modern field. Then, using three specific examples related to our research in arthropods, we evaluate the success of this approach.

One of the oldest insights into animal diversity is that variety can be partitioned and comprehended by grouping similar animals together. Discriminating similarities and differences among animals and using those to erect categories of distinct types of animals goes back at least to Aristotle and was a continuing thread in the natural sciences as they developed over the next two millennia. By the late 18th to early 19th

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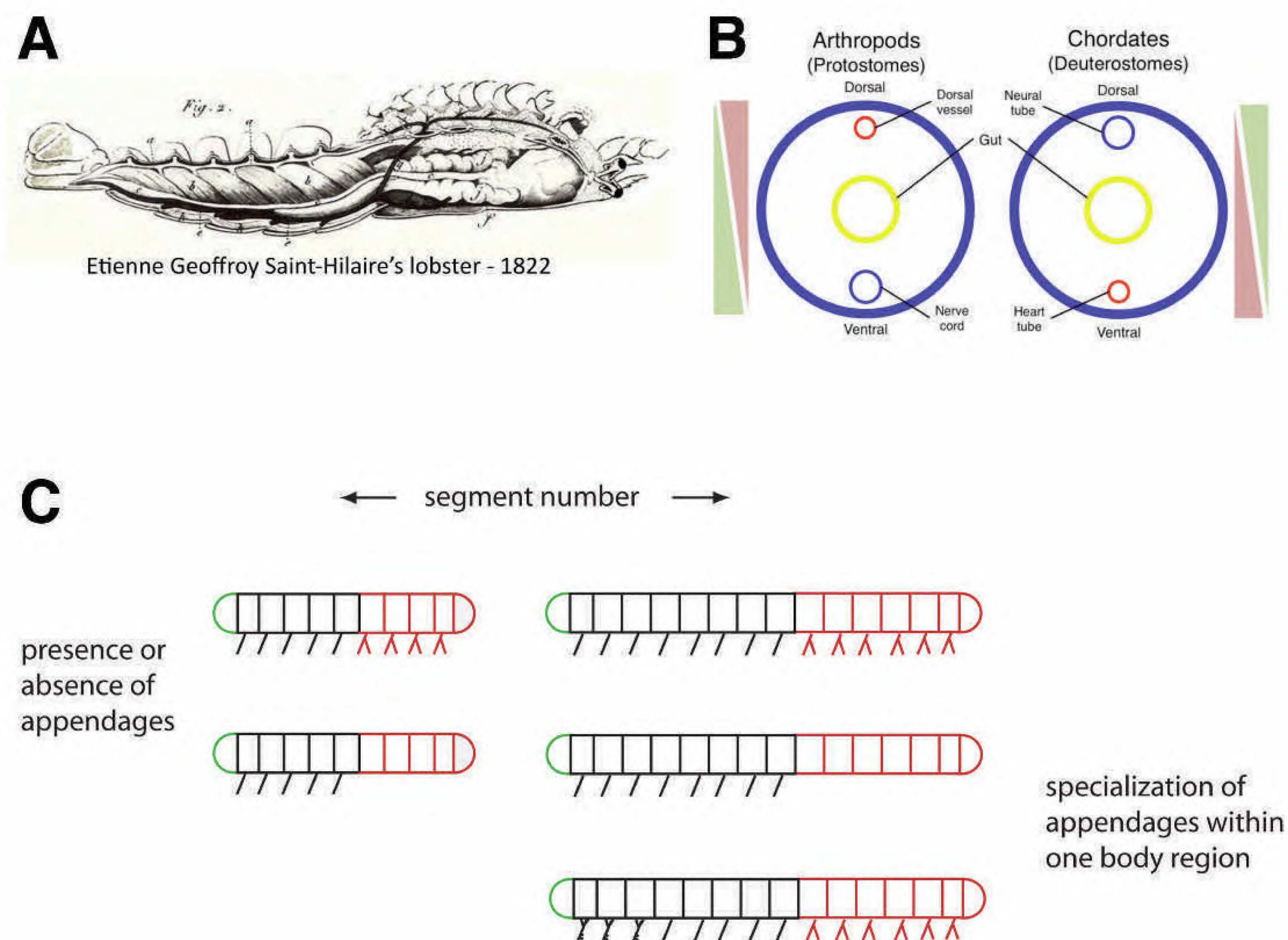


Figure 1. Examples of relationships of body plans discerned from morphological and molecular perspectives. —A. Geoffroy Saint-Hilaire's famous drawing of a lobster dissection “une coupe longitudinale du homard” from plate 7 (p. 119) of the 1822 article “Considérations générales sur la vertèbre.” In our Figure 1, the lobster is shown lying on its back, with its ventral nerve cord above the internal organs. In the inverted orientation herein, the body plan of the arthropod resembles that of the vertebrate. —B. Diagrammatic views of the protostome and chordate body plans. The dorsoventral structures occupy opposite sides of the body and are patterned by inverted domains of the diffusible growth factors (green gradient: *dpp*/BMPs) and their inhibitors (red gradient: *sog*/Chordin; from DV-axis-inversion, L'ontogenese, Wikipedia). —C. Generalized pattern of arthropod tagmatization. The diagram shows a simplified representation of the patterns of segmental diversification within arthropods: changes in segment number, presence or absence of segments on a particular segment, and specialization of appendages within one body region.

century, the conceptual framework that developed for this enterprise used the idea of homology (a structure similar under any transformation) versus analogy (any structure of similar function) to interpret parts of animals, and the similarities among animals were generalized using the conceptual model of an archetype (see Russell, 1917; Hall, 1994, 1999). The use of archetypes was a powerful tool for making comparisons highly explicit between taxa since the archetype was essentially a series of hypotheses about the morphology of a particular taxon. This theoretical framework, informed by a sophisticated grasp of the body plans of distinct taxa, led to specific discoveries (e.g., Goethe's discovery of the human intermaxillary bone) as well as broad sweeping theories (e.g., Geoffroy Saint-Hilaire's theory that vertebrates are essentially arthropods flipped onto their backs, or Richard Owen's demonstration via his vertebrate archetype that vertebrates are built from

repeated parts (Fig. 1A; Russell, 1917; Appel, 1987; Hall, 1999).

The theoretical framework of comparative morphology underwent a radical transformation at the end of the 19th and early 20th century due to two developments: Darwin's theory of evolution by natural selection and the modern synthesis of Mendelian genetics and population genetics (see Mayr, 1993; Gilbert et al., 1996; Bowler, 2003). The growth and predominance of these ideas had the effect of diverting the understanding of morphology from comparisons of form to a search for genetic (or other reductive) causes of form. In this new light, many of the hypotheses of the previous century disappeared. The conundrums of the past century were not resolved; they simply became unimportant in the new way of conceiving of natural phenomena. However, while old theories and hypotheses based on archetypes were not part of the modern research

agenda, they were still embedded in most textbooks dealing with animal diversity. For example, textbooks still presented generalized schemata to illustrate different phyla (see Brusca & Brusca, 2003) with traits of those schemata clearly defined (so-called true segmentation vs. pseudosegmentation vs. no segmentation). The persistence of these schemata was important to the emergence of EvoDevo as a field because they provided the background for comprehension of the importance of different fundamental body plans to metazoan diversity.

Developmental genetics made rapid progress in understanding the genetic regulatory control of external morphology in a few select model organisms. EvoDevo as a field gained momentum with the discovery that many of the genetic regulatory mechanisms that drive patterning in model organisms were broadly shared. A commonly cited example is *Pax6*, a gene for a transcription factor used to position the eyes in diverse organisms, even eyes that are not homologous (reviewed in Gehring, 2002). Carroll et al. (2001) postulated that a finite genetic toolkit existed for patterning embryos, and following on earlier ideas that changes in gene regulation were critical for phenotypic diversity (Jacob, 1977), popularized the idea that tinkering with this conserved set of regulatory genes could produce diverse body plans. This hypothesis reanimated some of the old questions about how the basic body plans of animals relate to one another. For example, a regulatory loop that patterned the dorsal axis of arthropods, but the ventral axis in vertebrates, resurrected Geoffroy's theory of vertebrates being upside-down arthropods (Fig. 1B; De Robertis & Sasai, 1996). From a gene's eye view, the genetic basis underlying morphological variation appeared to be remarkably similar throughout metazoans (see Carroll, 2005). This led to an initial enthusiasm that a modern, gene-based EvoDevo would resolve old issues of comparative morphology and ultimately be able to explain the great morphological radiations.

Here, we provide a brief review of progress on EvoDevo as viewed through the lens of examples relevant to our research dealing with the diversification of arthropods. In arthropods, a segmented body plan covered by a chitinous exoskeleton has produced evolutionary radiations of highly diverse and elaborated external morphologies. Many body segments bear appendages, and these are often specialized to perform distinct functions, both along the body axis in any particular species as well as between species. Much of arthropod diversity can be ascribed to segments and their appendages. In the vignettes that follow, we reflect on the power of the

EvoDevo approach to shed light on that diversity, and we conclude with reflections on why some questions have proved more tractable than others and what future approaches might include. We examine three features of variation of the arthropod body plan: (1) tagmatization and limb character along the anterior-posterior (A-P) body axis, (2) segment number, and (3) limb morphology. In each case, we outline the variation to be explained, the hypotheses generated from the genetic model system, the existing data, and what they explain.

TAGMATIZATION

VARIATION TO BE EXPLAINED

Arthropod diversity can be grossly characterized by variation in the numbers and specializations of segments. Most taxa have a fixed total number of segments, but some branchiopod crustaceans and some centipedes have a varying total number of segments. A feature common to all taxa is tagmatization, the regionalization of the body into distinct blocks of segments, namely, the head, thorax, and abdomen (Fig. 1C). Not only does total segment number vary between species, but also the number of segments in any particular body region can vary among taxa, e.g., the insect thorax has three segments, the decapod crustacean thorax has eight. Furthermore, overlaid on this divergence in tagma between distantly related taxa are modifications to tagma among even closely related taxa. For example, among decapods with eight thoracic segments (e.g., crabs, shrimp, lobsters) some might have all eight specialized for locomotion while others divide the eight into functional subspecialties. A common example is the appendages on the anterior thoracic segments that are modified to function with the head segments in feeding.

In general, the variation in arthropod segmentation can be grouped into three categories: (1) whether the total number of body segments is variable or fixed; (2) if it is fixed, how one taxon differs from another in total segment number and tagmatization; and (3) how body regions within a taxon are modified. These types of variation yield diverse patterns of segmentation among arthropods, patterns that have long been studied by naturalists (see Bateson, 1894; Lankester, 1904). Indeed, standard patterns of segmentation were so well known that in 1894 Bateson could catalogue instances of exceptions found in nature. These exceptions included a kind of variation in which one segment in a series assumed the character of another segment; Bateson called this phenomenon "homeosis." The recognition many years later that

homeosis could be caused by mutations of a single gene (Bridges & Morgan, 1923; Lewis, 1978) became a springboard for thinking about how patterns of segmentation might have evolved (Goldschmidt, 1940; King & Wilson, 1975; Lewis, 1978).

HYPOTHESES FROM DEVELOPMENTAL GENETICS

The discovery of the genetic basis of homeosis in flies by Lewis (1978) laid the foundation for understanding not only the developmental genetics behind segmental patterning in the dipteran *Drosophila melanogaster* Meigen, but also the evolutionary diversification of segment character. Using genetic analysis, the genes in the *Antennapedia* and *Bithorax* complexes of *D. melanogaster* were shown to control the development of the fruit fly body, with the exception of the termini (Lewis, 1978; Kaufman et al., 1980). The *Hox* genes have the unusual feature that their order along the chromosome mirrors their domains of function along the A-P body axis. This chromosomal linearity also suggests deep ancestral origins from multiple gene duplication events (Lewis, 1978).

Lewis discovered that the three posterior *Hox* genes in *Drosophila melanogaster* define the limbless abdominal body region. Given that the limbless abdomen is a defining feature of hexapods, Lewis (1978) speculated that the abdominal *Hox* genes originated at the base of the insect lineage through a serial gene duplication process. Arthropods with legs on all their trunk segments were predicted to lack these genes in their genomes, thereby avoiding the repression of limb development in the posterior region of their bodies. However, nearly all arthropods, as well as their closest relatives, the *Onychophora* Grube, or velvet worm phylum, have a full complement of *Hox* genes, and the simple hypothesis correlating new *Hox* genes with arthropod diversification was abandoned (Grenier et al., 1997; see below for discussion of why loss and gain of *Hox* genes as a plausible genetic change underlying arthropod diversification may yet be revived on a smaller scale). The next hypotheses correlating the evolution of tagma with *Hox* control of segment identity were built with the knowledge that nearly all arthropods have a full complement of *Hox* genes.

Once cloned, the *Hox* genes were identified as a family of transcription factors (McGinnis et al., 1984; Scott & Weiner, 1984) now recognized as a shared feature of multicellular animals. *Hox* genes are found throughout the Metazoa and are frequently clustered in the genome (reviewed in Lemons & McGinnis, 2006). Interestingly, less than 5% of the *Bithorax* locus identified by Lewis is devoted to the coding

sequence of the transcription factors. (Bender et al., 1983). These *cis*- regulatory regions scattered throughout the remainder of the locus contain binding sites for proteins that regulate the precise spatial and temporal expression of *Hox* genes. *Hox* genes function, in many animals, to pattern region-specific cell fates along the body axis. They realize this function by establishing specific expression domains along the body axis and regulating large suites of downstream target genes within these domains. Precisely how this results in the ultimate body plan of the animal is not completely known for any animal, although it is best understood in *Drosophila melanogaster*. Given the understanding of the *Hox* gene function in *D. melanogaster*, it was hypothesized that arthropod segmental diversity would correlate with changes in the regulation, both upstream and downstream of the *Hox* genes (Grenier et al., 1997). Evidence for intraspecific regulatory changes in *Hox* gene expression domains and in *Hox* gene targets followed in short order (for summary of regulatory changes in *Ubx*, see Barton et al., 2007). Below, we briefly recount some of the current data that support a model of how shifting *Hox* boundaries and *Hox* targets might explain how tagma evolved within crustaceans.

EXISTING COMPARATIVE DATA

Crustaceans use the last three of their five head appendages for feeding. However, in a number of taxa, appendages on anterior thoracic segments have been recruited to also function in feeding. For example, decapods have eight thoracic segments but only five pairs of locomotory limbs; the three anterior thoracic segments are modified for feeding. The first indication that the boundary between feeding and nonfeeding thoracic limbs might be under *Hox* control came from examining the expression of *Ubx* protein in crustaceans with various numbers of thoracic feeding limbs (Averof & Patel, 1997). Subsequent expression studies in isopod and branchiopod crustaceans supported this hypothesis (Abzhanov & Kaufman, 1999, 2000; Shiga et al., 2002). More recently, it was demonstrated that RNA interference (RNAi) silencing of *Ubx* in the peracarid crustacean, *Parhyale hawaiiensis* Dana, produces a decrease in the gene expression of *Ubx* relative to wildtype in the second and third thoracic segment. This decrease in expression causes a transformation of those limbs toward the feeding morphology of the first thoracic appendage (Liubicich et al., 2009). Conversely, ectopic expression of *Ubx* produces a transformation of feeding appendages toward more posterior limb morphologies (Pavopoulos et al., 2009). These functional results are consistent with

a model in which graded levels of *Ubx* protein control the character of limbs along the thorax: high levels of *Ubx* protein in the posterior thoracic limbs specify thoracic identity, whereas lower levels in anterior limbs specify a less elaborated, thoracic morphology. These are compelling results and provide a plausible model for re-specification and specialization of anterior thoracic appendages during crustacean evolution.

The paradigm of shifting boundaries is, however, only useful for a subset of the morphological transitions observed within the arthropods. Another body of evidence is accruing that suggests changes downstream of the *Hox* genes will play critical roles in other morphological transitions. For example, in flies, two *Hox* genes, *Ubx* and *abdA*, suppress limb development in the abdomen by direct repression of limb development genes (Vachon et al., 1992). The crustacean *Artemia* Leach (brine shrimp) expresses *Ubx/abdA* protein throughout the limb-bearing segments, but in *Artemia*, these genes do not appear to repress limb development. Interestingly, the *Artemia Ubx* gene is a weak repressor of the limb pathway, and differences in the translational product or amino acid sequence between *Artemia* and *Drosophila* correlate well with their respective strength of repression (Galant & Carroll, 2002; Ronshaugen et al., 2002; Shiga et al., 2002). In addition, it appears that the *Artemia abdA* mRNA is not translated into protein (Hsia et al., 2010). Thus, while progressively posterior boundaries of the *Bithorax* complex genes are maintained in this crustacean, they do not share the same regulatory targets as insects and do not regulate boundaries in limb morphology. In sum, *Hox* genes show a remarkable degree of conservation throughout the evolution of the Metazoa; at the same time, evolutionary changes in where they are expressed and in their specific functions can help explain evolutionary transitions in arthropod tagma.

WHAT IT DOES AND DOES NOT EXPLAIN

Of the patterns of segmental variability to be explained in arthropods, the *Hox*-based model addresses one pattern very well: how segments within a tagma are modified in a graded manner. The model does not address changes in segment number in any particular body region, i.e., how a taxon might evolve from having a thorax with 12 to eight segments. This is in part because the model focuses on limb morphology, yet tagma are not defined just by limb morphology but also by other segmental structures. The comparative analysis of *Hox* genes has also yielded the unexpected finding that in some species *Hox* genes do not pattern nonterminal regions of the

arthropod body plan. For example, while the posterior segments of the branchiopod crustacean, *Artemia*, do not bear limbs, no *Hox* gene expression has been detected in this region (Averof & Akam, 1995). Secondly, although the vast majority of morphological change occurs at boundaries between body tagma, in some cases taxa differ mid-tagma, e.g., the collembolan furca, which appears in the middle of the abdomen, from the fourth abdominal segment. (The furca is a fused, forked appendage that gives the name of springtails to collembolan insects.) This region is not at an expected boundary of the *Hox* genes and has not (as yet) been shown to be associated with any novel boundaries of *Hox* genes. This is related to another, much more common phenomena not encompassed by this model. Numerous crustaceans have larval stages with patterns of appendages quite distinct from their adult stages. Specifically, they show differences in segmentation patterns at sequential stages of the lifecycle that do not consist of graded changes at boundaries. Candidates for the genetic control of these morphological transitions are not yet obvious.

SEGMENT NUMBER

VARIATION TO BE EXPLAINED

Segment number varies among the greater than million species of arthropods, ranging from hundreds in some millipedes (Enghoff et al., 1993) to eight in ostracod crustaceans (Schram, 1986). Interestingly, most classes and orders of arthropods do not vary in segment number (with a few noteworthy exceptions, e.g., the geophilomorph centipedes; Minelli & Bortoletto, 1988), and segment number is a defining character for some major lineages. Unfortunately, the paradigm of using *Drosophila melanogaster* as a starting point for hypotheses about the genetic/developmental control of a morphological character is not possible with the case of segment number. Insects do not vary, for the most part, in total segment number, and dipteran insects form their segments in a highly derived manner. Mutations that increase segment number have not been identified in *D. melanogaster*. However, vertebrates show lineage-specific diversity in segment number and share with arthropods the ancestral mode of adding segments sequentially during development. Therefore, we use models of segment development from vertebrates to consider variation in segment number among arthropods. This comparison is supported not only by the shared fact of sequential segment addition but also by the finding that a number of the regulatory genes that

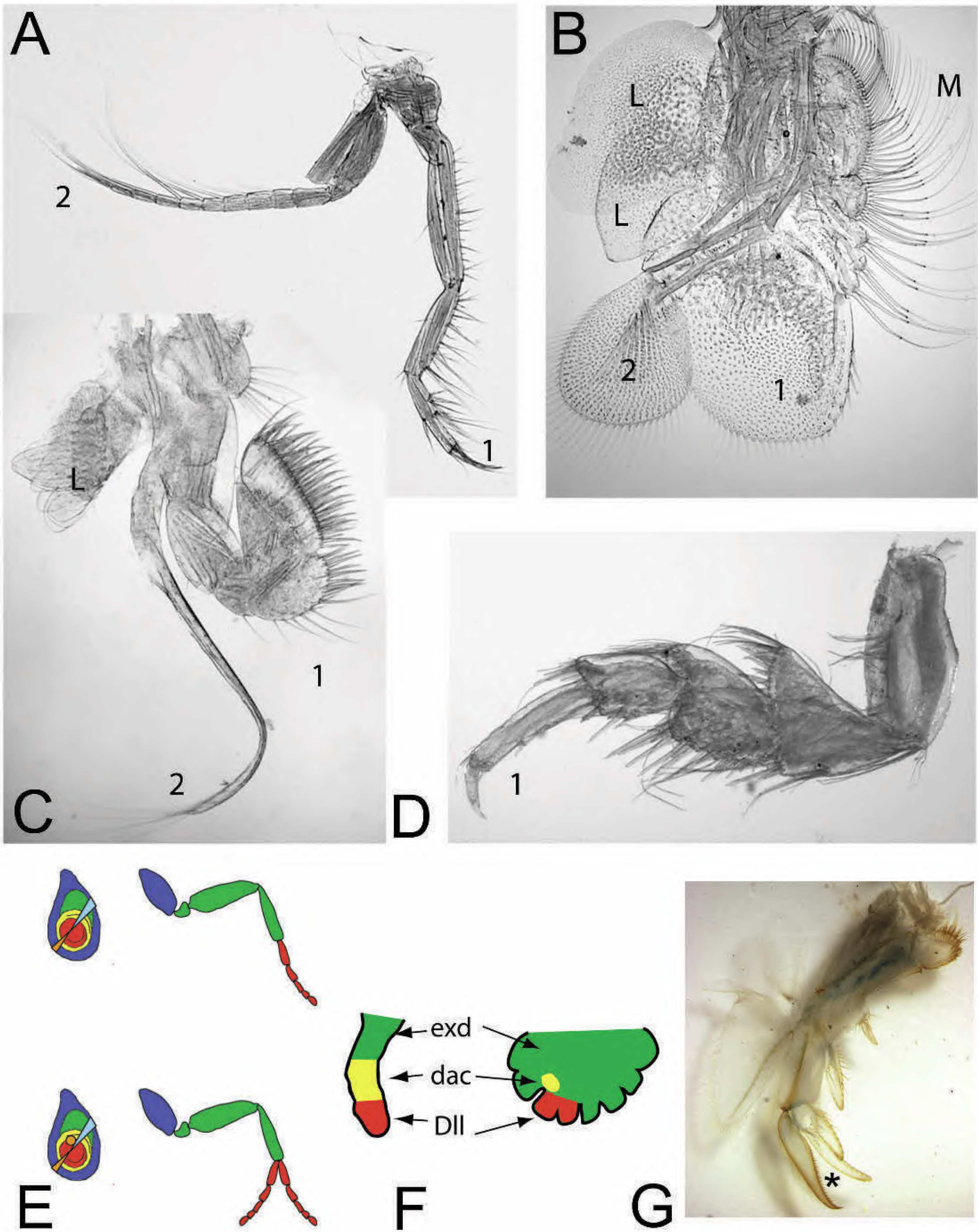


Figure 2. A–D. Variety in arthropod limb morphology, using crustacean examples. —A. Thoracic limb of the mysid shrimp, *Americamysis bahia* Molenock. —B. Thoracic limb of the fairy shrimp, *Thamnocephalus platyurus* Packard. —C. Anterior thoracic limb (maxilliped) of the grass shrimp, *Paleomonetes pugio* Holthuis. —D. Thoracic limb of the isopod, *Cirolina concharum* Lat. The two main branches are labeled 1 and 2, with additional lobes labeled as medial (M) or lateral (L). Note that beyond the fundamental variability in number of branches and lobes, limb parts are highly variable in terms of shape, proportion, and setal numbers and morphology. —E. Patterning in the leg disc of *Drosophila*. Diagram at top indicates signaling along the A-P segment boundary, which initiates PD outgrowth of the leg. The genes that establish the PD axis—*Distal-less* (red), *dachshund* (green), and *extradenticle/homothorax* (blue)—are activated in circular domains in the larval leg disc by a combination of signals (gold, light blue). As the larval leg disc grows and extends into the adult leg, these genes function to pattern three domains along the PD axis of the leg (diagram at bottom). Experimentally initiating new PD outgrowths by misexpression of a signal (gold) gives rise to artificially branched legs. F, G. Comparative expression data in limbs of varying

operate in vertebrate segmentation play similar roles in arthropods (Peel et al., 2005).

HYPOTHESES FROM DEVELOPMENTAL GENETICS

In vertebrates, segments arise from embryonic somites, which develop sequentially in a head-to-tail direction in the embryo (Fig. 1D). Somites bud off from the anterior presomitic mesoderm, an unpatterned region of active growth in the posterior of the embryo. Somites form at a species-specific rate, e.g., 30 min./segment in zebrafish, 90 min./segment in chickens, and 120 min./segment in mice (Romanoff, 1960; Tam, 1981; Schröter et al., 2008). At the same time, cells are added to the posterior of the presomitic mesoderm through the process of gastrulation, thereby allowing for continued development of segments. The size and number of somites depend on a dynamic interaction between three factors: the size of the presomitic mesoderm, the position of a posteriorly moving wavefront of determination, and oscillations of certain genes known as the segmentation clock (Fig. 1E). In general, in those vertebrate species examined, all use a similar molecular toolkit to run the segmentation clock: mainly genes of the *Notch*, *FGF*, and *Wnt* signaling pathways. In mice and fish, mutations in the oscillator genes cause severe defects in the somites. These pathways function to make pulses of signaling molecules in the presomitic mesoderm (Cooke & Zeeman, 1976; Elsdale et al., 1976; Palmeirim et al., 1997; Dubrulle et al., 2001; Sawada et al., 2001). Each cycle of the oscillator converts oscillations in time to a periodic pattern in space and results in the appearance of a pair of segments.

Gomez et al. (2008) asked whether the evolutionary variation in segment number between snakes, mice, chicken, and zebrafish could have resulted from developmentally varying either the size of the presomitic mesoderm, the position of the determination wavefront, or the periodicity of the segmentation clock. They found that in snakes, which have a high number of segments, the rate of oscillation of the segmentation clock was high relative to the growth and elongation of the presomitic mesoderm. Thus, snake embryos segment the presomitic mesoderm faster than other vertebrates, making smaller and

more numerous somites. Do arthropods similarly control the size of segments generated per cell generation in the region of the embryo that forms segments to control segment number? As we point out below, understanding of the fundamental cellular processes of segmentation in arthropods lags behind that of vertebrates. Consequently, understanding the genetic control of segment number in arthropods is only just emerging.

EXISTING COMPARATIVE DATA

In most arthropods, segments form in an A-P progression (Sander, 1976; Minelli & Fusco, 2004; Peel et al., 2005). However, there are surprisingly few data that indicate whether segments form with a species-specific periodicity since patterns of segment addition are typically described only with reference to morphological stage and not developmental time. The assumption is that segment addition is regular and, in examining some crustaceans, we have found that segments are added with linear periodicity (Williams et al., 2012). Whether a regular periodicity in segment addition is widespread in arthropods remains unknown.

The combination of three features that control sequential segment addition in vertebrates—a growth zone, a determination wavefront, and a segmentation clock—has not been demonstrated for any arthropod. In general, most sequentially segmenting arthropods have a region of unpatterned tissue in the posterior that generates segments, i.e., a growth zone. However, the extent of the unpatterned tissue and its rate of growth or depletion during the process of segment addition are completely unknown. There is no evidence as of yet for a determination front in arthropods, at least of the kind found in vertebrates that is regulated by antagonistic gradients of signaling molecules. However, a growing body of literature suggests that the molecular toolkit that runs the vertebrate segmentation clock is conserved in spiders, sequentially segmenting insects, and recently, we have found evidence for the function of clock orthologs in crustaceans (Williams et al., 2012). In each of these cases, *Notch* signaling has been demonstrated to play a role in the proper formation of sequentially added segments.

←
morphologies. —F. Simplified schematic of gene expression in limbs of two crustaceans. The genes that establish the PD axis include *Distal-less* (red), *dachshund* (yellow), and *extradenticle/homothorax* (green) and are expressed in a pattern similar to *Drosophila* (at left) and *Porcellio scaber* Latreille (at right). —G. In *Triops longicaudatus* LeConte, the three genes are expressed even in the unusually shaped limb bud of this species that develops into a highly modified (phyllopodous) limb form. The asterisk marks the two distal branches in the schematic of the limb bud and the adult limb.

WHAT IT DOES AND DOES NOT EXPLAIN

The discovery that *Notch* signaling plays a role in sequentially segmenting arthropods was initially hailed to indicate that vertebrate and arthropod segmentation was homologous. Closer comparison shows that, even among arthropods, disruption of the *Notch* signaling network has variable effects. In some species like *Drosophila melanogaster*, mutations in *Notch* signaling have no consequences for segmentation. While the discovery of a role for *Notch* signaling in arthropod segmentation is significant, a robust model of *Notch* function as well as the possibility that *Notch* signaling serves as a molecular oscillator remains unresolved. Thus, whether the segment number is regulated in arthropods via a balance between clock rate and rate of growth in the posterior awaits further research.

LIMB MORPHOLOGY

VARIATION TO BE EXPLAINED

The array of limb structures in arthropods is truly astounding (Fig. 2A–D; Brusca & Brusca, 2003). Even apparently simple, cylindrical limbs, like the walking legs of a crab, may have elaborate lateral outgrowths (in this case, functioning as gills and hidden beneath the carapace). Beyond cylindrical walking legs, arthropod limbs show adaptations for swimming, grasping, sensing, food handling, and many other functions. Correspondingly, limbs may be flattened into paddles, calcified for crushing pincers, or adorned with elaborate setal arrays. It is tempting to organize all arthropod limbs as variations on a theme of a single limb axis with medial or lateral outgrowths. However, it is not clear that this characterization is evolutionarily accurate, since some would argue that the ancestral limb had two fundamental branches (Walossek, 1993, 1999; Boxshall, 2004). In one major arthropod group, in extant Crustacea, the limb has two branches, i.e., the main axis is bifurcated. Thus, to understand some of the variation in limb morphology, there are at least three main questions to be addressed: (1) what patterns the main axial outgrowth; (2) how is the main limb axis bifurcated; and (3) what patterns the vast array of medial and lateral outgrowths that occur proximally on the limb axis? In addition to these main categories of variation, there are numerous features of appendages that differ widely between limbs, both within and between taxa, e.g., setal type and number, cuticular thickness and specialization or jointing, etc. This variation is often crucial to functional differences between limbs and also needs to be explained.

HYPOTHESES FROM DEVELOPMENTAL GENETICS

In *Drosophila melanogaster*, limb primordia are positioned at the boundary that defines the posterior portion or compartment of each segment. Subsequently, via signaling activated at the A-P compartment boundary, limb axes are defined and proximo-distal (PD) elongation occurs. The gene network involved in patterning the PD leg axis is well described (Fig. 2E–G; reviewed in Nagy & Williams, 2001; Angelini & Kaufman, 2005b). In short, the leg is divided into three domains along the PD axis and patterned by genes with mutually exclusive gene expression domains. Loss of these gene expression domains causes loss of position-specific leg tissue and truncated or shortened legs. Experiments in *D. melanogaster* demonstrated that it was possible to partially duplicate the PD axis; manipulating signaling along the A-P boundary formed new sites of PD elongation and ultimately branched legs with duplicated distal axes (Struhl & Basler, 1993; Diaz-Benjumea et al., 1994). This led to the hypothesis that reiterating the PD patterning network along the A-P segment boundary could have generated naturally occurring, branched limbs (Campbell & Tomlinson, 1995).

EXISTING COMPARATIVE DATA

Based on the hypothesis above, limb patterning genes from *Drosophila melanogaster* were candidates to regulate limb patterning in other species. When *D. melanogaster* genes were examined in other species, both in expression and function, it became clear that, while some genes are expressed similarly across arthropods, the entire network from *D. melanogaster* was not conserved. Critically, the *wingless* and *decapentaplegic* genes that function directly upstream of PD elongation in *D. melanogaster* do not show conserved function, even within insects, and so cannot explain modulation of PD elongation (Angelini & Kaufman, 2005a, 2005b). PD elongation is based primarily on the activation of *Distal-less* in *D. melanogaster*. Although analyses of *Distal-less* function in other arthropods show that it is required for PD growth of limbs (Beerman et al., 2001; Shoppmeier & Damen, 2001; Khila & Grbic, 2007), there is no evidence that the PD patterning network is reiterated to form branches. Instead, in every case where it has been examined, the evidence points to a single PD patterning axis whether the limb has only one axis, a bifurcated axis, or is a highly modified paddle (Fig. 2G; Williams, 1998; reviewed in Williams & Nagy, 2001; Williams et al., 2002).

Indeed, the network of genes regulating PD outgrowth is broadly conserved.

WHAT IT DOES AND DOES NOT EXPLAIN

Two points in limb development appear broadly conserved with little variation. First, all limbs examined are positioned along the A-P segment boundary. In spite of this conserved positioning, the signaling that subsequently occurs along the A-P boundary that initiates PD outgrowth in *Drosophila melanogaster* is not conserved. Nevertheless, once it is initiated, the network of PD leg patterning and elongation is the second broadly conserved aspect of leg patterning. This appears to be the case even in limbs of highly divergent morphology, like the flattened, multilobed paddles of branchiopod crustaceans. The deep conservation of PD patterning is striking and probably represents a core set of genes that, once activated, can produce a limb (i.e., a limb-patterning module). However, one core aspect of variation in limbs, branches or outgrowths from the main axis, is not explained by the comparative data. The analysis of candidate genes yielded no patterns that gave rise to new hypotheses explaining branching, and we currently have no good working models to account for such limb variation.

The analysis of candidate genes from *Drosophila melanogaster* limb patterning is complicated by the fact that a number of genes involved in limb patterning have pleiotropic effects. For example, *Distal-less* protein is found in almost every appendage examined to date, but evaluating its role in patterning limb outgrowth is confounded by its additional role in sensory development (Mittmann & Scholtz, 2001; Williams et al., 2002; Williams, 2008). Although *Distal-less* is well known to function in the nervous system of *D. melanogaster* (Panganiban, 2000), its role in limb patterning is distinct both spatially and temporally because of *D. melanogaster*'s specialized metamorphic mode of development, where the segregation and patterning of cells fated to become limbs occur much earlier than the differentiation of limb sensory structures. Most arthropods lack this segregation between limb patterning and limb differentiation, and, therefore, gene expression regulating limb patterning overlaps gene expression regulating sensory patterning, confounding the inference of function in limbs with complex morphology.

CONCLUSIONS

EvoDevo promised to revive and answer some longstanding questions about the morphological diversity that results from adaptive radiations. How

far has the paradigm of diversification using a finite toolkit taken us within the field of arthropod EvoDevo? With respect to the three areas analyzed herein, each feature shows surprising instances of deep conservation of certain patterning mechanisms. For tagmatization and limb identity along the A-P axis, *Hox* genes play a fundamental role in shifting the boundaries of limb morphology. For variation in segment number, *Notch* involvement is widespread. For limb morphology, there is a highly conserved PD patterning module.

The idea of a finite toolkit has proven to be a surprisingly robust hypothesis. In many cases, when we examine nonmodel organisms, we find the same regulatory genes in the same roles they play in model systems. Furthermore, some aspects of variability can be explained by changes in broadly conserved genes. For example, the subspecialization of limbs within tagma seems to be well modeled by shifts in the boundaries of *Hox* expression. However, what has also become clear is that there are two main drawbacks to following this approach. First, candidate genes may not be widely conserved or may have pleiotropic effects that complicate our modeling their roles. Second, and more profound, the level of patterning that is deeply conserved is often not the level that establishes the details of morphology that are fundamental to adaptive radiations. This is most clearly illustrated by the analysis of limbs. Whereas there does seem to be a widespread PD patterning module that forms a single PD axis in all limbs, we have no general models to explain the morphological variability that characterizes the functional diversification of limbs. Nor do we know how the limb axis is bifurcated in two-branched forms. Given that the branching and outgrowths of limbs are the very substrate of their functional diversity, this conserved patterning module has not served us well in analyzing that diversification.

Even as we write this, EvoDevo is being transformed by the development of new tools that in part address some of the limitations. Transgenesis and RNAi are transforming our ability to conduct functional studies in nonmodel arthropods. These methods will facilitate a much-needed wider taxon sampling. With RNAi, we can test gene function in nonmodel systems and test whether the assumptions derived from a few model systems hold more broadly. High-throughput sequencing/proteomics speed up gene discovery at an incredible rate and are limited only by financial resources rather than the life history strategies of an organism. The sequenced genomes confirm that the generic developmental toolkit is widely present. Interestingly, 30%–60% of open

reading frames identified in the arthropod species sequenced to date have no identifiable orthologs in other species, and as much as 50% of the active transcriptome in some species are these orphan genes. These orphan genes may have important functions in nonmodel systems. These new approaches are opening new windows on how genomes evolve. How directly those windows will lead to more a complex understanding of the kinds of changes that natural selection can act upon remains to be seen.

In addition to the increased use of genomics, transcriptomics, and proteomics to further our mechanistic understanding of development, several other parallel trends or research programs can be discerned (e.g., Müller, 2007). One is a trend toward using computational and bioinformatics approaches to understand development and how developmental processes evolve; another is the integration of ecological and environmental aspects of developmental biology into what is often called EcoDevo (Gilbert, 2001). In addition, evolution and development studies applied to smaller-scale evolutionary problems within insects have had success (e.g., Kopp, 2011; Prud'homme et al., 2011; Frankel et al., 2012). Finally, it is sometimes forgotten that another important trend is continued emphasis, especially in marine invertebrates (Love, 2009), on developing a phylogenetically informed comparative embryology.

We conclude that EvoDevo has made great strides in uncovering the common features underlying the development of morphology. The field has also made a start at the discovery of both protein and regulatory changes that correlate with morphological changes. At the same time, resolution of many longstanding questions about morphological diversity has largely not occurred. Arthropod morphological diversity is structurally complex and not often captured by broadly conserved patterning elements. The ultimate goal is to understand the complete arc from genetic regulation to expressed phenotype in a set of related organisms. This goal of understanding how the genotype translates into the complex, evolvable morphological phenotype remains for the future.

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